Final Scientific Report IS-3802-05

Cover Page

BARD Project Number: IS-3802

Date of Submission of the report: 28.05.1010

Project Title: A Novel Approach to Mycotoxin Detoxification in Farm Animals Using Probiotics Added to Feed Stuffs

Investigators		**Institutions**
Principal Investigator (PI):	**R. Shapira**	**Hebrew University**
Co-Principal Investigator (Co-PI):	**J.M. Grizzle**	**U. Tennessee, USA**
Collaborating Investigators:	**C. Mendis-Handagama**	**U. Tennessee, USA**
	M. Pines	**ARO, Min. Ag.**
	N. Paster	**ARO, Min. Ag**

Keywords: detoxification, trichothecene, probiotic, T-2, Tri101,

Abbreviations commonly: GFP = green fluorescent protein

Budget: IS: $206,120 US: $158,880 Total: $365,000

_____ _____

Signature Signature

Principal Investigator

Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation				
Invited review papers				
Book chapters				
Books				
Master theses			2	
Ph.D. theses			1	
Abstracts			1	
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings				
Longer Visits (Sabbaticals)				

Description Cooperation:

Probiotic strains containing Tri101 generated in Israel were sent to the USA

Plasmid constructs containing the Tri 101 gene were sent to Israel

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract:
T-2 toxin, a toxic product belongs to the trichothecene mycotoxins, attracts major interest because of its severe detrimental effects on the health of human and farm animals. The occurrence of trichothecenes contamination is global and they are very resistant to physical or chemical detoxification techniques. Trichothecenes are absorbed in the small intestine into the blood stream. The hypothesis of this project was to develop a protecting system using probiotic bacteria that will express trichothecene 3-O-acetyltransferase (Tri101) that convert T-2 to a less toxic intermediate to reduce ingested levels *in-situ*. The major obstacle that we had faced during the project is the absence of stable and efficient expression vectors in probiotics. Most of the project period was invested to screen and isolate strong promoter to express high amounts of the detoxify enzyme on one hand and to stabilize the expression vector on the other hand. In order to estimate the detoxification capacity of the isolated promoters we had developed two very sensitive bioassays. The first system was based on *Saccharomyces cerevisiae* cells expressing the green fluorescent protein (GFP). Human liver cells proliferation was used as the second bioassay system. Using both systems we were able to prove actual detoxification on living cells by probiotic bacteria expressing Tri101. The first step was the isolation of already discovered strong promoters from lactic acid bacteria, cloning them downstream the Tri101 gene and transformed vectors to *E. coli*, a lactic acid bacteria strain *Lactococcus lactis* MG1363, and a probiotic strain of *Lactobacillus casei*. All plasmid constructs transformed to *L. casei* were unstable. The promoter designated lacA found to be the most efficient in reducing T-2 from the growth media of *E. coli* and *L. lactis*. A prompter library was generated from *L. casei* in order to isolate authentic probiotic promoters. Seven promoters were isolated, cloned downstream Tri101, transformed to bacteria and their detoxification capability was compared. One of those prompters, designated P201 showed a relatively high efficiency in detoxification. Sequence analysis of the promoter region of P201 and another promoter, P41, revealed the consensus region recognized by the sigma factor. We further attempted to isolate an inducible, strong promoter by comparing the protein profiles of *L. casei* grown in the presence of 0.3% bile salt (mimicking intestine conditions). Six spots that were consistently overexpressed in the presence of bile salts were isolated and identified. Their promoter reigns are now under investigation and characterization.

Achievements: The detoxification capacity of two probiotic strains of *Lactobacillus casei*, each having different promoter upstream the Tri101 gene, were examined by HPLC analysis. Bacteria were grown overnight, washed and concentrated by resuspension in 1:80 of the original volume in PBS buffer. Aliquots were taken at different time intervals and the amount of the mycotoxin T-2 (belongs to the trichothecenes family) was analyzed by HPLC. Detoxification was more efficient in constructs having promoter 101 than in those having promoter 104 (Fig. 1).

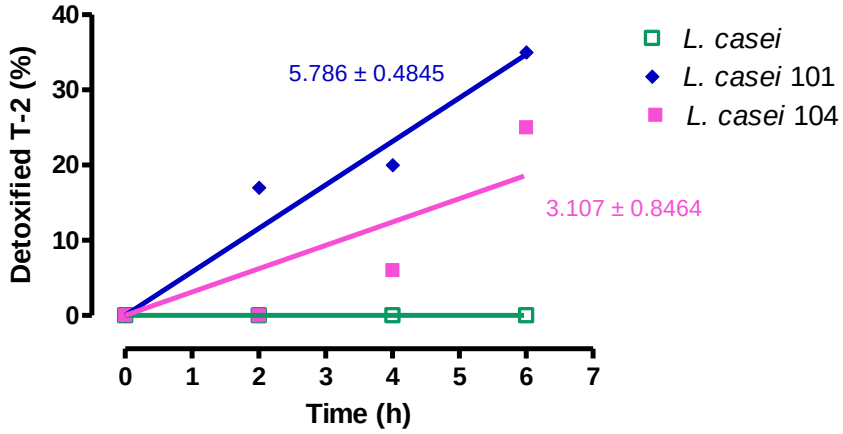


Figure 1. T-2 detoxification by chimeric *L. casei*. ▫ represent wild type *L. casei*. ■ represent *L. casei* harboring the pCU*tri*104 plasmid. ♦ represent *L. casei* harboring the pCU*tri*101 plasmid.

The detoxification capability of the two strains was also examined in a bioassay using *Saccharomyces cerevisiae* expressing the green fluorescent protein (GFP). Yeast cells were grown to mid-log and then exposed to filtered bacterial supernatant after six hours of incubation with T-2. The detoxification rate obtained by the probiotic bacteria harboring P101 promoter was significantly higher from the strain having the P104 promoter (Fig. 2).

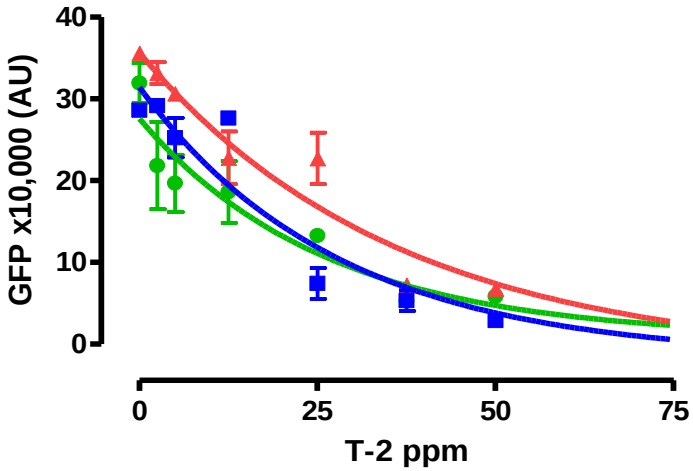


Figure 2. GFP fluorescence of *Saccharomyces cerevisiae* exposed to upper soup of *L. casei* (●), *L. casei* + pCU*tri*101 (▲) and *L. casei* + pCU*tri*104 (■) incubated with different concentration of T-2.

Human liver cells proliferation was used as second bioassay system for determination of T-2 detoxification. Huh-7 cell line were exposed to the upper soup of detoxifying bacteria (p101*Tri*), upper soup of promoter-less bacteria (p100*Tri*) was used as controls. Viable cells were counted by microscopic observation after exposure of 48 h. The most significant detoxification effect obtained at 0.01µM of T-2 as illustrated in Fig. 3.

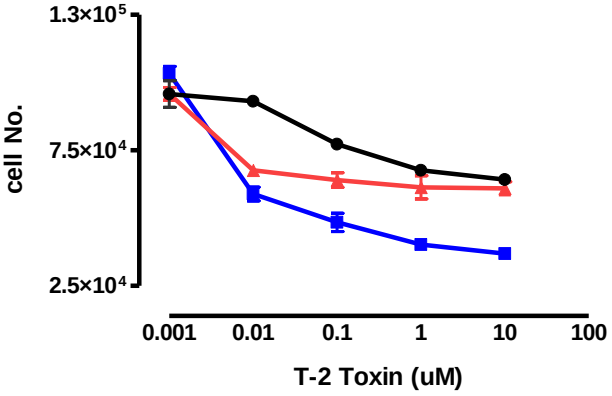


Figure 3: Huh-7 human liver cells proliferation bioassay for T-2 detoxification. Square (■) represents the T-2 toxin. Triangle (▲) represents upper soup of MG1363 containing the promoterless *Tri*101 (p100*Tri*). The diamond (♦) represents upper soup bacteria containing the *Tri*101 under the control of the *lacticin* RM promoter (p101*Tri*).

We have shown in both bioassays the detoxification capability by bacteria harboring the p101*Tri* plasmid. According to the obtained results we conclude that Huh-7 human liver cells were 10 times more sensitive to T-2 than *Saccharomyces cerevisiae*. The conversion product of T-2 toxin obtained of the detoxifying bacteria was less toxic than T-2 for both yeast and liver cells.

In order to improve the expression of the Tri101 gene in the lactic acid bacteria, we carried out two approaches: a) testing known promoters of lactic acid bacteria; and b) isolating native promoters of *L. casei* while focusing on inducible promoters that operate under intestinal environment conditions.

The published promoters: *ldh* (8), 23 (10), lacticin RM-103 (4), *slp*A, (7)and *lac*A (1), were isolated from chromosomal DNA of the relevant bacteria by PCR using the published sequences and cloned into pCU pasmid. The detoxification capacity of the five promoters cloned lactic acid bacteria strain *Lactococcus lactis*, was examined by HPLC analysis (Fig .4.)

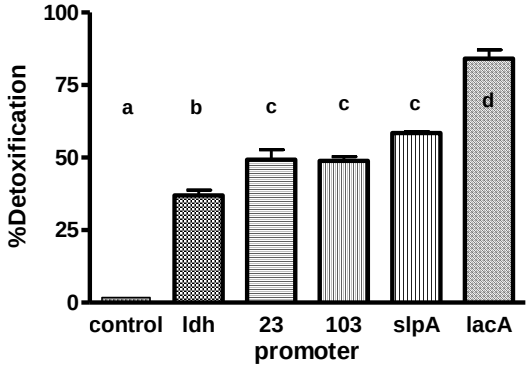


Figure 4: T-2 toxin detoxification by the lactic acid bacteria *L. lactis* MG1363, under the control of different promoters. Different letters indicate statistical significance between results. Control is MG1363 containing the promoter-less *tri* with T-2 toxin.

Represent bacteria expressing Tri101 gene under the control of lactate dehydrogenase promoter (*ldh*). Represent bacteria expressing Tri101under the control of P23 promoter. Represent Tri101 expression under the control of lacticin RM promoter (P103). Represent Tri101expression under the control of the S-layer protein promoter (*slp*A). Represent Tri101expression under the control of the

inducible promoter *lac* (Tuky's test, $P \leqslant 0.01$). The most pronounced detoxification ability was obtained when *Tri*101 was cloned upstream *lac*A promoter.

The second approch was isolation of authentic probiotic promoters. Having a high efficiency transformation system is an indispensable tool in order to "fish out" promoters. Minor modifications of Posno *et. al.* 1991 (9) method yielded a very efficient transformation rate: more than million transformants per 1µg DNA could be obtained even with plasmids larger than 9 kbp (Table 1).

Table 1. The effect of plasmid size on transformation efficiency.

plasmid size	6.5 kbp	6 kbp	7 kbp	7.2 kbp	8.2 kbp	9.2 kbp
transformants per 1µg DNA	1.75×10^5	7.4×10^6	8×10^6	4×10^6	2×10^6	2.19×10^6

In order to obtain promoter library from *Lactobacillus casei* 393 DNA fragments (0.5-1.5 kb) were cloned into the promoter-screening vector pBV5030, which contains a promoterless chloramphenicol acetyltransferase gene (6) Seven fragments were identified based on their ability to confer resistance against chloramphenicol in *Escherichia coli*. After challenging with elevated concentration of chloramphenicol, three isolates (p201, p153 and p41) that showed consistent resistant were selected for further analysis

To compare their promoter strengths, an *E. coli-lactococcal* shuttle vector pARGFP containing a GFP gene as the reporter gene was constructed (Fig 5). The expression of GFP by three putative promoter regions were compared (Fig. 6). The putative promoter p201 exhibited the highest promoter activity both in *E. coli* JM109 (figure 6) and in *L. lactis ssp. cremoris* MG1363 (not shown).

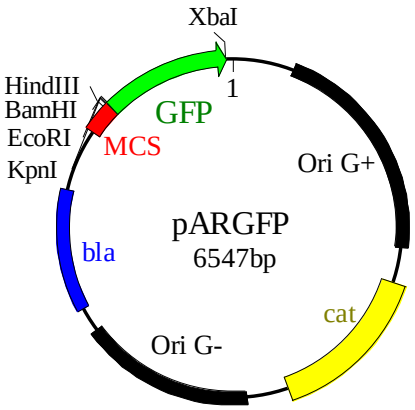


Figure 5. Schematic map of the shuttle vector pARGFP. The ori of Gram negative and Gram positive bacteria are represented G-, G+ respectively. The selection for gram negative is by ampicillin and for Gram positive by chloramphenicol . represent the promoterless GFP gene.

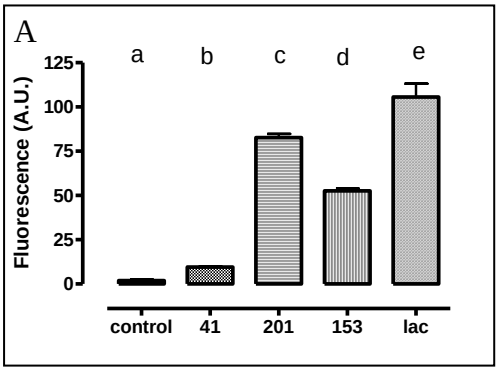
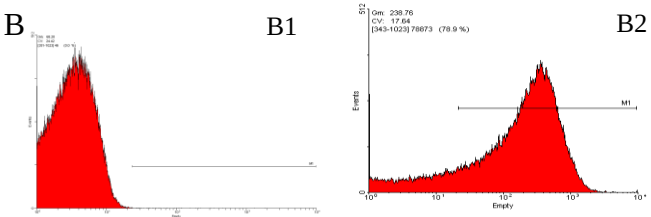
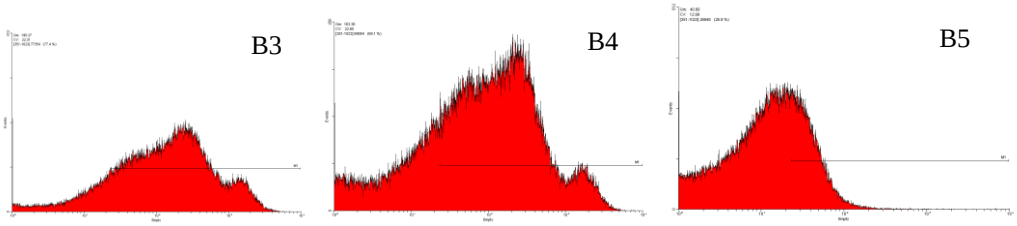


Figure 6: Promoters strength comparison.

GFP fluorescence of the *E. coli* transformants containing pAR series plasmids with putative promoters (A). *E. coli* GFP fluorescence as shown by FACS, B1-negetive control, B2-GFP under the *lac* promoter, B3- GFP under the P201 putative promoter, B4-GFP under the P153 putative promoter, B5-GFP under the P41 putative promoter (B). Relative GFP fluorescence units are the mean standard deviations of 6 measurements performed in two independent experiments. Different letters represents statistical difference (student's t test α<0.05).





Two isolates, p201 and p41, showed the highest and the lowest activity, were chosen for farther investigation RACE (rapid amplification of cDNA ends) method was applied to find the transcription initiation site and DNA sequencing revealed the consensus region recognized by the sigma factor (figure 6).

P201

CGATTTAGGCATCAGACTTGCGATAAAGCGGAACCCCTGCTACAATTGGTTCTGTTGGTA
AGGGAAAACAGCAAATGTTGGATTCCTAATTTTTTTCAGCTTGCTGGGTCGGAAAACGAC
TCGGAGGGACGTGGAATGACCCATTAAAGAAGGACGTGCCTTATGCATAC
****

P41

CACGAAAATGGCGAAGCATTGTCCCACGCCTTTAACGGGTACTTGGCGCCAGTAATCGTCA
GGTCGGTGATAGGGGTTAAATTGACAACTGCCACATAGCGATAATCTGGCAGCGGTTGAAT
TTCATGGACACCCGGGGCATAGTAATCGACCCGATTCTGCACATCCAAAAGGTGAATGCGTT
******

Figure 6: Nucleotide sequences of the putative promoters from *Lactobacillus casei* 393. Putative promoter sequences are underlined (-35 region, (…..); -10 region, (___); transcription initiation sites are indicated in green color; SD sequences are indicated with asterisks underneath the nucleotides. Putative TG-dinucleotide is indicated in blue.

The intestine conditions enable differential protein expression in bacteria colonizing the small intestine (14). In order to isolate induced and strong promoter that will enable expression of the detoxifying protein TRI101 in probiotic bacteria, we investigated the protein profile of the probiotic bacteria *Lactobacillus casei* exposed to small intestine conditions including 0.3% bile salt compared to bacteria that wasn't exposed to bile salt by two dimensional electrophoresis gel. *L. casei* was grown at anaerobic conditions to $O.D._{600}$~1, cells were washed and resuspended in MRS pH=7 and exposed to 0.3% bile salt for 4 hours. Comparing the protein distribution between cells exposed to bile extract to unexposed cells revealed consistently 10 spots that increased in their intensity in the bile-exposed cells. This analysis was repeated in 3 independent experiments. We have selected five spots that showed increased abundance for MALDI TOF analyzed (Fig 8). The predicted proteins names and their functions are summarized in Table 2. Non of the isolated proteins could be attributed directly to the exposure of the bacterium to bile salt.

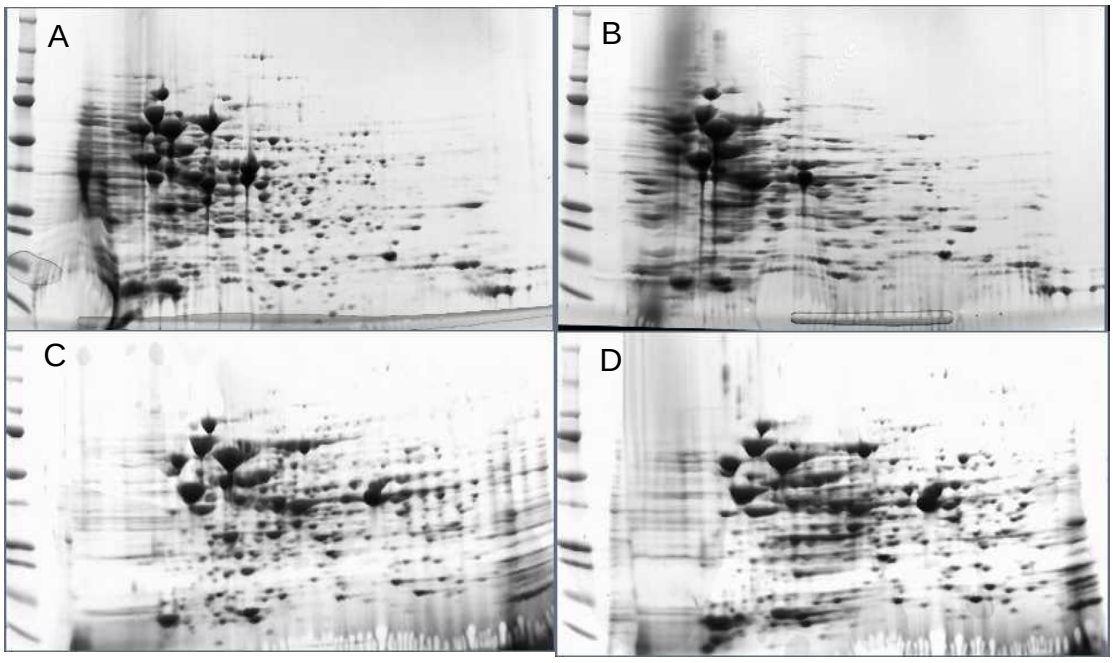


Figure 7: Two dimensional electrophoresis gel patterns of the probiotic bacteria L. casei, A-control not exposed to bile salt pI 3-11. B- Exposed to 0.3% bile salt pI 3-11. C-contol not exposed to bile salt pI 4-7. D- exposed to 0.3% bile salt pI 4-7.

Table 2: The sequenced spots, their function and their function in gram positive bacteria.

Protein Name	Protein Function	Gram positive
EscU	assembled to allow for the direct transmission of bacterial virulence effectors into the host cell	Not mentioned in the literature in gram positive bacteria.
Enoyl-ACP (acyl carrier protein) reductase	responsible for catalyzing the final step in each elongation cycle of bacterial type II fatty acid biosynthesis (FAS II), and plays a key role in regulation of the pathway.	A product of the FabI gene in *Bacillus anthracis*. A key NADH-dependent enzyme in FAS II (type II fatty acid synthase system) that catalyzes the final and rate determining step of chain elongation (13).
GalE (UDP-galactose 4-epimerase)	One of the key genes involved in Leloir pathway, the pathway catalyses the transformation of galactose into glucose 1-phosphate	uced in the presence of galactose and repressed by glucose. The promoter galPI is located immediately upstream of the operon and is induced in the presence of galactose. This promoter directs transcription of the galT, gaIE, and galK genes. The second promoter, gaLP2, is located within the operon just upstream of the gaIE gene. This promoter is responsible for constitutive transcription of the galE and galK genes. Comparison of the S. lividans gal operon to the Escherichia coli gal operon indicates the presence of a constitutive promoter positioned upstream of galE in both operons (2).
Mannose / fructose-specific component IIA	The bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS) is a multi-protein system involved in the regulation of a variety of metabolic and transcriptional processes. necessary for the uptake of carbohydrates across the cytoplasmic membrane and their phosphorylation	In both Gram-positive and Gram-negative bacteria proteins of PTS are involved in carbon catabolite repression. They also control the induced expression of several catabolic operons in response to inducer availability by modifying the activities of transcriptional regulators, transport proteins and enzymes (11).
LuxS	The *luxS* gene encodes S-ribosylhomocysteinase, an enzyme which generates the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) from S-ribosyl- L –homocysteine. DPD gives rise to several furanone derivatives, often collectively referred to as AI-2 (Quorum sensing molecule). Plays a key role in bacterial cell-cell communication.	LuxS-dependent signal play an important role in the biofilm formation of *Streptococcus mutans* bacterial communication fulfills an important role in coordinating various processes in the oral cavity (5). Strongly induced by low pH in *Lactococcus lactis* (3).. Growth phase dependent with maximal expression in the mid exponential, and salt dependent (12).

Attempts are now in progress to clone the upstream region of the selected *L. casei* proteins that were differentially expressed during exposure to 0.3% bile salt.

References:

1. **de Vos, W. M., and E. E. Vaughan.** 1994. Genetics of lactose utilization in lactic acid bacteria. FEMS Microbiol Rev **15:**217-37.
2. **Fornwald, J. A., F. J. Schmidt, C. W. Adams, M. Rosenberg, and M. E. Brawner.** 1987. Two promoters, one inducible and one constitutive, control transcription of the Streptomyces lividans galactose operon. Proc Natl Acad Sci U S A **84:**2130-4.
3. **Frees, D., F. K. Vogensen, and H. Ingmer.** 2003. Identification of proteins induced at low pH in Lactococcus lactis. Int J Food Microbiol **87:**293-300.
4. **Hindre, T., J. P. Le Pennec, D. Haras, and A. Dufour.** 2004. Regulation of lantibiotic lacticin 481 production at the transcriptional level by acid pH. FEMS Microbiol Lett **231:**291-8.
5. **Huang, Z., G. Meric, Z. Liu, R. Ma, Z. Tang, and P. Lejeune.** 2008. luxS-Based Quorum-Sensing Signaling Affects Biofilm Formation in Streptococcus mutans. J Mol Microbiol Biotechnol.
6. **Jeong, D. W., Y. C. Choi, J. M. Lee, J. H. Kim, J. H. Lee, K. H. Kim, and H. J. Lee.** 2006. Isolation and characterization of promoters from Lactococcus lactis ssp. cremoris LM0230. Food Microbiol **23:**82-9.
7. **McCracken, A., M. S. Turner, P. Giffard, L. M. Hafner, and P. Timms.** 2000. Analysis of promoter sequences from Lactobacillus and Lactococcus and their activity in several Lactobacillus species. Arch Microbiol **173:**383-9.
8. **Oozeer, R., J. P. Furet, N. Goupil-Feuillerat, J. Anba, J. Mengaud, and G. Corthier.** 2005. Differential activities of four Lactobacillus casei promoters during bacterial transit through the gastrointestinal tracts of human-microbiota-associated mice. Appl Environ Microbiol **71:**1356-63.
9. **Posno, M., R. J. Leer, N. van Luijk, M. J. van Giezen, P. T. Heuvelmans, B. C. Lokman, and P. H. Pouwels.** 1991. Incompatibility of Lactobacillus Vectors with Replicons Derived from Small Cryptic Lactobacillus Plasmids and Segregational Instability of the Introduced Vectors. Appl Environ Microbiol **57:**1822-1828.
10. **Que, Y. A., J. A. Haefliger, P. Francioli, and P. Moreillon.** 2000. Expression of Staphylococcus aureus clumping factor A in Lactococcus lactis subsp. cremoris using a new shuttle vector. Infect Immun **68:**3516-22.
11. **Reizer, J., S. Bachem, A. Reizer, M. Arnaud, M. H. Saier, Jr., and J. Stulke.** 1999. Novel phosphotransferase system genes revealed by genome analysis - the complete complement of PTS proteins encoded within the genome of Bacillus subtilis. Microbiology **145 (Pt 12):**3419-29.
12. **Sewald, X., S. H. Saum, P. Palm, F. Pfeiffer, D. Oesterhelt, and V. Muller.** 2007. Autoinducer-2-producing protein LuxS, a novel salt- and chloride-induced protein in the moderately halophilic bacterium Halobacillus halophilus. Appl Environ Microbiol **73:**371-9.
13. **Tipparaju, S. K., D. C. Mulhearn, G. M. Klein, Y. Chen, S. Tapadar, M. H. Bishop, S. Yang, J. Chen, M. Ghassemi, B. D. Santarsiero, J. L. Cook, M. Johlfs, A. D. Mesecar, M. E. Johnson, and A. P. Kozikowski.** 2008. Design and synthesis of aryl ether inhibitors of the Bacillus anthracis enoyl-ACP reductase. ChemMedChem **3:**1250-68.
14. **Yuan, J., B. Wang, Z. Sun, X. Bo, X. Yuan, X. He, H. Zhao, X. Du, F. Wang, Z. Jiang, L. Zhang, L. Jia, Y. Wang, K. Wei, J. Wang, X. Zhang, Y. Sun, L. Huang, and M. Zeng.** 2008. Analysis of host-inducing proteome changes in bifidobacterium longum NCC2705 grown in Vivo. J Proteome Res **7:**375-85.